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THE EFFECTS OF HEAVY METAL IONS ON THE MOTILITY OF SEA URCHIN SPERMATOZOA[1]

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Intense activity of sea urchin spermatozoa follows dilution in sea water. The high performance level is of limited duration; metabolism and motility decline to a low steady state and fertilizing capacity diminishes progressively with time (Tyler and Tyler, 1966). This so-called "dilution effect" has been attributed to the action of trace amounts of heavy metal ions present in seawater on the respiratory enzymes of the spermatozoa (Rothschild and Tuft, 1950; Rothschild and Tyler, 1954). Although there are numerous reports on the effect of various metal ions on the O_2 consumption of sea urchin spermatozoa, quantitative data on the action of heavy metals on spermatozoan motility are not generally available.

Rothschild and Tuft (1950) reported that small amounts (4×10^{-5} M) of $CuCl_2$ or $ZnCl_2$ added to dense suspensions ($> 4 \times 10^8$ sperm/ml) of *Echinus esculentus* spermatozoa increased their level of O_2 consumption, but had no effect on the respiration of dilute suspensions ($< 4 \times 10^8$ sperm/ml). While these authors did not provide actual measurements, they stated that neither copper nor zinc had any effect on the swimming speed of the spermatozoa.

Mohri (1956) found that $CuCl_2$ or $ZnCl_2$ at low concentration (10^{-5} M) accelerated the O_2 uptake of *Hemicentrotus pulcherrimus* spermatozoa, but at a higher concentration (10^{-4} M) inhibited it. According to Barron, Nelson, and Ardao (1948), 5×10^{-6} M $HgCl_2$ stimulates the respiration of *Arbacia punctulata* spermatozoa, but a concentration of 10^{-4} M inhibits their O_2 consumption. These investigators did not relate the effects on O_2 uptake to the motile activity of the cells.

Additional evidence for the sensitivity of sea urchin speramtozoa to the toxic effects of heavy metal ions comes from experiments on the ability of chelating agents to prolong both the motility and fertilizing capacity of sea urchin spermatozoa. Rothschild and Tyler (1954) found that 10 micromolar ethylenediaminetetraacetic acid (EDTA) depressed dilution-induced increase in O_2 uptake and delayed senescence of *Echinus esculentus* spermatozoa. Tyler (1953) used a variety of agents which bind heavy metal ions—amino acids, diethyldithiocarbamic acid (DEDTC), 8-hydroxyquinoline (oxine), and α-benzoinoxime (cupron)—to prolong the life span and fertilizing capacity of sea urchin and sand dollar spermatozoa. Mohri (1956) also found that chelating agents would suppress the respiratory increases observed on dilution of *Hemicentrotus pulcherrimus* sperm. The investigators concluded that these agents act to depress respiration, and thereby prolong sperm motility and viability, by binding heavy metals ordinarily present in the seawater.

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Although it is often assumed that increased O_2 consumption by the spermatozoon is accompanied by increased motile activity and decreased O_2 consumption by decreased motile activity both Robbie (1946) and Rothschild (1948) have demonstrated that spermatozoan motility is not a simple function of oxygen uptake.

In this report, using a convenient and objective determination of swimming speed, we explore quantitatively the effects of alterations in the environmental concentrations of heavy metal ions on the motile activity of *Arbacia punctulata* spermatozoa.

Materials and Methods

Spermatozoa were collected from sea urchins, *Arbacia punctulata*, by injecting 0.53 M KCl into the perivisceral cavity. The shed sperm were concentrated at $1000 \times g$ in a clinical, table top centrifuge for 3 minutes, the supernatant fluid removed, and the packed sperm stored at 4° C. Immediately prior to the motility rating tests, samples of the spermatozoa were diluted to a final concentration of $10^7 \pm 10\%$ sperm/ml of filtered seawater, determined by optical density measurements of the sperm cell suspensions (Nelson, 1972). This concentration was selected empirically because it fell within the optical density range appropriate to the motility determinations.

Five milliliters of seawater suspensions of spermatozoa were added to round colorimeter tubes containing either: (1) 0.5 ml distilled water as control, (2) 0.5 ml 1% formaldehyde to kill the cells, or (3) 0.5 ml of the reagent being tested. The contents were mixed thoroughly by inverting twice.

The swimming speed of the spermatozoa was determined by the centrifuge-orientation, optical density method using a Bausch and Lomb Spectronic 20 Colorimeter at a wavelength of 540 nm and a clinical centrifuge. Measurements of sperm cell density were made immediately after mixing the sperm suspensions into the reagents and after each of three four-minute centrifugations at $120 \times g$. The method depends on the fact that, under the force generated in a mild gravitational field, the normally, slightly positively geotropic *Arbacia* sperm cells become oriented centrifugally and swim with decreased randomness to the bottom of the tube. While the dead cells also become similarly oriented, they no longer swim and are only minimally sedimented at $120 \times g$. Therefore, the rate of decrease of optical density (ΔOD), which was previously determined to be linearly proportional to the swimming speed, may be calculated after correction for any slight displacement of non-motile cells, and in comparison to the ΔOD of the untreated control cells, viz., $M = \Delta OD_x / \Delta OD_c \times 100$. Changes in the speed of the swimming sperm cells are expressed in terms of per cent of control swim rate.

All experimental procedures were carried out in an air-conditioned room (22–23° C) and initial swimming speeds were obtained within 5 minutes of dilution of the spermatozoa in filtered seawater. Branham (1966) and Timourian and Watchmaker (1970) have reported that the motile activity of sea urchin spermatozoa remains stable for approximately 30 minutes after dilution in seawater. The reagents tested included: $CuCl_2$ 500 nM to 100 μM, $ZnCl_2$ 5 μM to 10 mM, $MnCl_2$ 5 μM to 10 mM, $HgCl_2$ 10 nM to 1 mM, EDTA 10 μM to 5 mM (adjusted to pH 7.8 with NaOH before use). The natural concentrations, in μEq liter, of

TABLE I

Dose- and time-dependence of zinc effects. Motility, expressed as per cent of control motility (100%), of Arbacia punctulata *spermatozoa after each of a series of three 4-minute centrifugations at 120 × g; $10^7 \pm 10\%$ sperm in 5 ml filtered Woods Hole seawater; temperature 22–23° C. Each value represents the mean of individual determinations made on spermatozoa collected from three different* Arbacia punctulata, *± standard deviation*

Zinc added in μEq/liter	% Control motility		
	Centrifugation #1	Centrifugation #2	Centrifugation #3
2500	22 ± 5.6	20 ± 5.6	20 ± 4.8
500	36 ± 2.2	34 ± 2.8	30 ± 2.8
250	44 ± 2.6	29 ± 0.6	17 ± 4.1
50	80 ± 7.2	52 ± 5.8	30 ± 4.6
25	155 ± 10.8	111 ± 4.6	97 ± 8.7
5	113 ± 5.0	105 ± 7.1	107 ± 10.6
2.5	98 ± 3.6	101 ± 1.0	100 ± 1.0

certain heavy metal ions in seawater (using data adapted from Richards, 1972) are zinc—0.3, copper—0.1, manganese—0.1, and mercury—0.002.

RESULTS

Zinc

Figure 1 shows the response of *Arbacia punctulata* spermatozoa to increases in environmental zinc. Sea water contains approximately 0.3 μEq zinc/liter (Richards, 1972) ; increasing the zinc concentration to 5 μEq/liter causes little more than a 10% increase in swim speed while a tenfold increase in zinc concentration (to 25 μEq/liter) speeds up the sperm by 55%. Increasing the amount of environmental zinc to 50 μEq/liter slows the swimming speed of the spermatozoa to 20% below control values. As the concentration of zinc is increased from 250 to 500 to 2500 μEq/liter of seawater, motility declines from 44% to 36% to 22% of control levels. However, we had to increase the concentration of zinc in the medium to 15 mEq/liter in order to immoblize the sperm cells completely.

Prolonged contact with zinc ion, even at concentrations which initially stimulate motility, has an apparently debilitating effect on the motile activity of the spermatozoa. Table I shows that, following the addition of 25 μEq of zinc, *Arbacia* sperm cells swim at 155% of the control speed after the first four minutes centrifugation, 111% of the control swim rate after the second four-minute centrifugation. At 250 μEq zinc/liter, motility declines from 44% to 29% to 17% of the control speed. In both higher (0.5 to 2.5 mEq) and lower (2.5 to 5 μEq) concentrations of added zinc, there is no significant change in the swimming speed of the *Arbacia* spermatozoa after the first 4 minutes centrifugation.

Copper

The concentration of copper in seawater is equivalent to about 0.1 μEq/liter. The effect of increases in environmental copper on the swimming speed of sperma-

tozoa collected from each of two sea urchins is illustrated in Figure 2. Addition of 0.5 μEq copper speeds the sperm by approximately 10%. In the presence of 2.5 μEq added copper, sperm motility falls to 86 or 66% of control values, decreases to 37 or 58% of control values in 5.0 μEq excess copper, to 31 or 36% of control values in 25 μEq excess copper, and ceases completely in the presence of 50 μEq

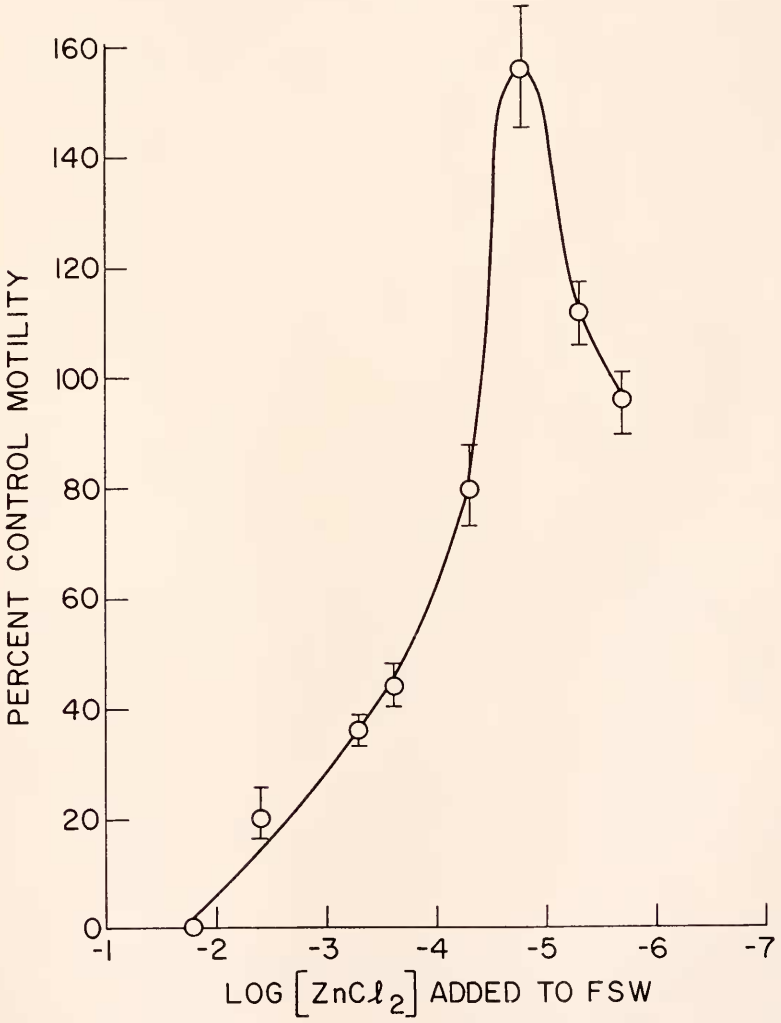

FIGURE 1. Effect of $ZnCl_2$ on the swimming speed of *Arbacia punctulata* spermatozoa. Varying concentrations of $ZnCl_2$ added to filtered Woods Hole seawater containing $10^7 \pm 10\%$ *Arbacia punctulata* spermatozoa elicit a biphasic dose-dependent response. *Abscissa* represents the zinc chloride concentrations added, in log moles per liter seawater/sperm suspensions; *ordinate* is the swim rate of treated sperm expressed as per cent of the control rate in seawater. The points and ranges indicate the mean and standard deviations of individual determinations made on spermatozoa collected from three individual *Arbacia*. The incubation varied from 22–23° C on different days.

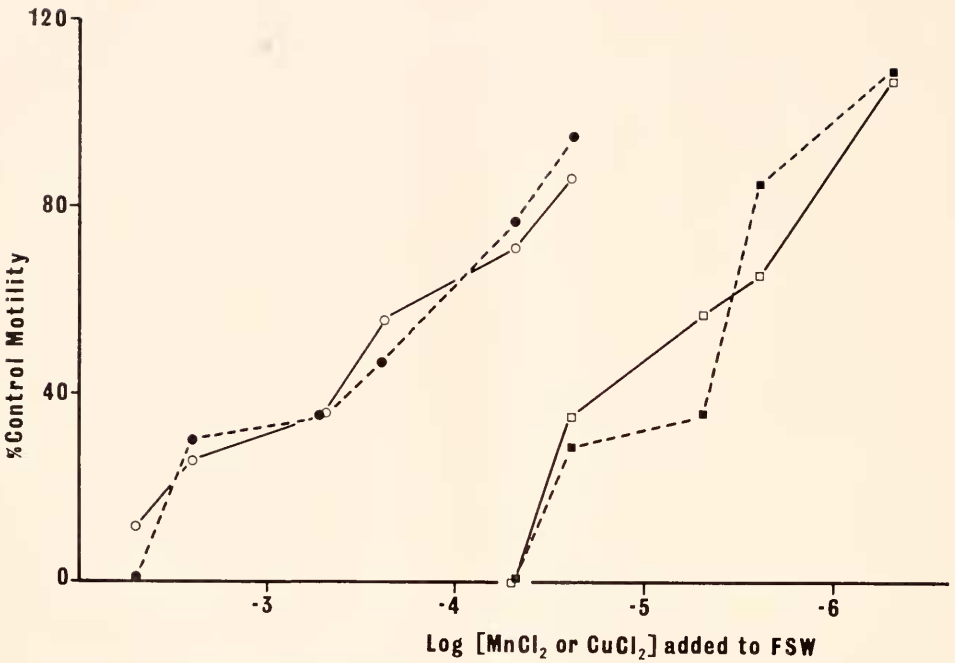

FIGURE 2. Effects of $CuCl_2$ and $MnCl_2$ on the swimming speed of *Arbacia punctulata* spermatozoa. $MnCl_2$ and $CuCl_2$ added to filtered Woods Hole seawater containing $10^7 \pm 10\%$ *Arbacia punctulata* spermatozoa decreased their swimming speed in proportion to the concentration. *Abscissa* is concentrations of metal ion added, in log moles per liter of seawater/sperm suspensions: copper—open and closed squares, manganese—open and closed circles (open and closed data points from replicate runs). *Ordinate* is the swim rate of treated sperm expressed as per cent of control rate in a seawater. Each point represents an individual determination of swim rate expressed as a per cent of control rate in seawater at a temperature of 22–23° C. Note that while the seawater content of Mn^{2+} and Cu^{2+} both equal about 10^{-7} equivalents per liter, the sperm cells are more sensitive to excess copper by about two orders of magnitude.

excess copper. Copper appears to exert its effect on sperm motility almost immediately after addition to the sperm cell suspensions; further significant declines in swimming speed do not occur after the first 4 minutes centrifugation.

Manganese

Woods Hole seawater contains approximately the same concentration of manganese as it does copper. But as shown in Figure 2, manganese exerts much less of an inhibitory effect on *Arbacia* sperm motility than does copper. Addition of 0.5, 2.5 or 5 μEq manganese/liter to the sperm cell suspensions does not measurably affect the swimming speed. Excess manganese up to 25 μEq/liter causes only a 4 or 15% reduction in motility and further increases result in only a slow decline in Arbacia sperm swimming activity; in the presence of 2.5 mEq manganese/liter—approximately three orders of magnitude greater than the normal seawater concentrations of this heavy metal ion—motile activity is still about one-quarter of the control value.

Mercury

The effect of $HgCl_2$ on the motility of *Arbacia punctulata* spermatozoa was measured after incubation of the cells with mercuric ion for fixed intervals of time. Figure 3 (solid line) is an example of the changes in motility of sperm cells collected from a single sea urchin after 8 minutes incubation in 5×10^{-8} to 10^{-3} M/l $HgCl_2$. A marked increase in swim speed is observed at mercury concentrations between 10^{-4} and 10^{-7} M. In 5×10^{-6} M $HgCl_2$ *Arbacia* sperm swim at about 270% of the control rate. In higher concentrations of mercury, motility declines but is still 10% above control values in 10^{-3} M $HgCl_2$. As the concentration of mercuric ion is decreased below 10^{-7} M, acceleration gradually declines and the

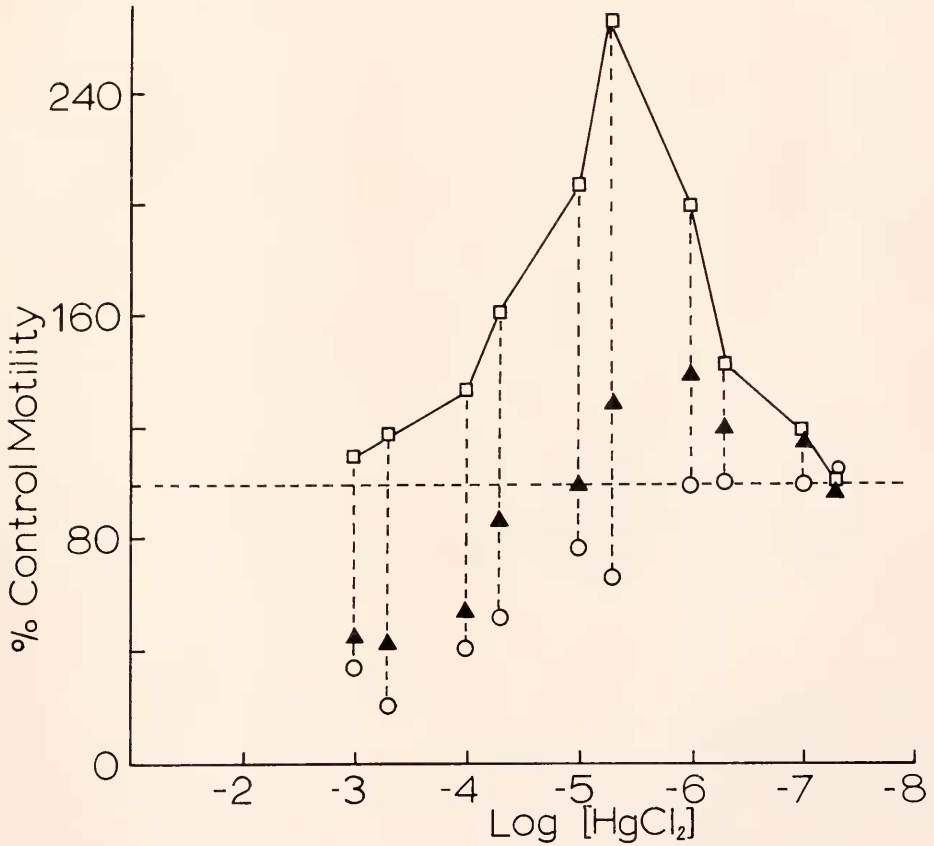

FIGURE 3. Effect of $HgCl_2$ on the swimming speed of *Arbacia punctulata* spermatozoa. The $HgCl_2$ added to filtered Woods Hole seawater containing $10^7 + 10\%$ *Arbacia punctulata* spermatozoa shows pronounced dose-dependent and time-dependent effect. The *abscissa* is the log molar concentration of mercury in seawater/sperm suspensions: open square—8 minutes incubation; solid triangles—16 minutes incubation; open circles—24 minutes incubation. The *ordinate* shows the swim rate of treated sperm expressed as per cent of control rate in seawater, temperature 22–23° C.

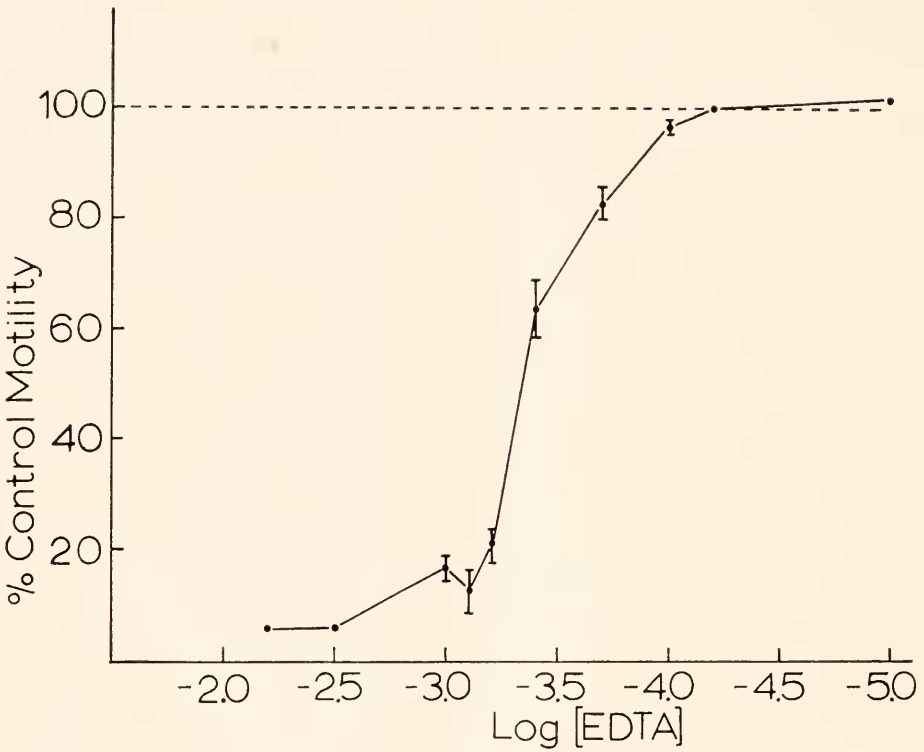

FIGURE 4. Effect of EDTA on the swimming speed of *Arbacia punctulata* spermatozoa. EDTA added to filtered Woods Hole seawater containing $10^7 + 10\%$ *Arbacia punctulata* spermatozoa per milliliter depresses the swimming speed at effective concentrations. The *abscissa* is in log molar concentration of EDTA in seawater/sperm suspensions. The *ordinate* is the swim rate of treated sperm expressed as per cent of the control rate in seawater. The points and ranges represent the mean and standard deviations of separate determinations made on spermatozoa collected from three individual *Arbacia punctulata*. Incubation temperature was 22–23° C.

effect is negligible at 5×10^{-8} M. Moreover, the stimulatory effect of $HgCl_2$ on *Arbacia* sperm motility is transitory. The dashed lines in Figure 3 show the decline in swim speed observed after 16 and 24 minutes incubation in various concentrations of $HgCl_2$. For example, in 5×10^{-6} M $HgCl_2$ motility is 170% above control level after the first centrifugation (8-minute measurement), 30% above control level after the second centrifugation (16-minute measurement) and 33% below control level after the third centrifugation (24 minutes measurement).

Since an initial stimulation and subsequent decline in sperm motility was observed at all concentrations of $HgCl_2$ tested, the effect of preincubation with mercuric ion on sperm swimming speed was determined. The results of a ten-minute preincubation of the sperm cells in 10^{-3} to 10^{-7} M $HgCl_2$ essentially confirm the observations recorded on sperm cell delayed response illustrated in Figure 3.

EDTA

EDTA, in excess of 100 μM/l of seawater, exerts a profoundly depressant effect on the swimming speed of sea urchin spermatozoa (Fig. 4). Motility was measured in concentrations of EDTA ranging from 5×10^{-3} to 5×10^{-5} molar. In Figure 4 we note the following: in seawater suspensions containing less than 10^{-4} Moles of EDTA per liter, motility appears normal. A twofold increase in EDTA concentration, to 2×10^{-4} M, causes a 17% decrease in the swimming speed of the spermatozoa. Another doubling of the EDTA concentration, results in a further decline in motility to 64% of control values. In 6×10^{-4} M EDTA swim speed declines to only 21% of control rates. As the concentration of EDTA is further increased, the motility of the *Arbacia* spermatozoa continues to decline at a slow rate and in 2×10^{-3} M EDTA the sperm are virtually immotile.

Discussion

From the evidence we may conclude that the motile activity of *Arbacia punctulata* spermatozoa is affected in a concentration-dependent and, in some instances, time-dependent, manner by the heavy metal ion composition of the environment.

Although we are not prepared at present to specify the particular sites within the spermatozooon on which these metal ions exert their action, previous studies indicate that the effects on motility may in part depend on heavy meal interaction with protein sulfhydryl groups to form mercaptides. Barron *et al.* (1958) suggested that soluble —SH groups are necessary for the activity of enzymes essential for spermatozoan viability.

Sea urchin spermatozoa exhibit a biphasic response to increases in the environmental concentration of zinc ions. A three- to thirtyfold increase of the zinc in the seawater causes the *Arbacia punctulata* spermatozoa to swim between 13 and 55% faster than control sperm in normal seawater. This acceleration of the swim rate is only transitory since, within 30 minutes of the addition of 5 to 25 μEq zinc/liter to the seawater in sperm suspensions, motility has declined to control levels. Larger excesses of zinc, above 50 μEq/liter, do not stimulate sperm motility, but instead cause an immediate fall in swimming speed which continues to decline as the time of incubation in zinc ion is increased. Zinc, at a concentration of 25 μEq/liter, which according to Rothschild and Tuft (1950) has no effect on the oxygen uptake of dilute suspensions of *Echinus* spermatozoa, exerts a short-term stimulatory effect on the motility of *Arbacia* spermatozoa. Therefore zinc is probably acting in other ways than only by combining with the soluble sulfhydryl groups involved in regulation of sperm cell metabolism. Morisawa and Mohri (1972) found that both the sperm tails and isolated microtubules of the sea urchin *Pseudocentrotus depressus* appear to concentrate zinc, and they concluded that this ion may play an important role in the contractile process. The fairly large excess of zinc needed to overcome the initial acceleration of *Arbacia* sperm motility indicates that, at concentrations above 50 μEq/liter, zinc's inhibitory effect may be due to binding at active sites on contractile proteins. In fact, Utida and coworkers (1956) report that other divalent cations, Cd^{2+}, Co^{2+} and Ni^{2+}, increase ATP hydrolysis by washed sperm tails of the sea urchin *Heliocidaris crassispina* at 1 mM/l, while Cu^{2+} and Zn^{2+} activate the enzyme at 10^{-4} molar but inhibit at

the higher concentration. Our data on swimming speed (see Fig. 1) bear a most striking resemblance to the graphic representation of Utida, Maruyama and Nanao (1956) relating sperm tail apyrase activity to the zinc concentration.

In the range that we examined, both copper and manganese ions inhibit *Arbacia* sperm motility, but while we found copper to be about 100 times more potent an inhibitor of sea urchin sperm swimming than was managanese, still, at the lowest concentration—0.5 μM—$CuCl_2$ actually stimulates motility by about 10%. An average hundredfold increase above environmental manganese causes an 8% decrease in swim speed while an equivalent increase in the copper content of seawater depresses *Arbacia* sperm motility by 77%. The comparatively large amounts of manganese which must be added to the sperm cell suspensions before significant changes in motile activity can be detected suggest that manganese may, among other things, have only limited access to active groups which are important for propulsive activity. Alternatively, we cannot overlook the report of Garbers, Lust, First and Lardy (1971) that the sperm cells of the sea urchin, *Stronglyocentrotus* are remarkably endowed with guanyl cyclase, and to a lesser extent, adenyl cyclase, both of which are highly Mn^{2+} dependent. Agents which influence cyclic nucleotide metabolism affect both motility and respiration of spermatozoa according to Garbers *et al.* (1971).

Since small amounts of copper (2.5 μEq liter) in the seawater/sperm suspensions cause a measurable decrease (34%) in swim speed, it seems likely that copper may act at essential regulatory sites within the spermatozoan flagellum. The role of copper in metabolism is well documented; excess copper ions exert their effects at a variety of enzymatic sites. Morisawa and Mohri (1972) believe that cytochrome C oxidase in the midpiece of sea urchin spermatozoa accounts for most of the copper found in these sperm while Barnes and Rothschild (1950) claim that sea urchin sperm are able to bind copper to the extent of 300 times the amount in seawater. Rothschild and Tuft (1950) have shown that copper arrests the decline in oxygen consumption of dilute suspensions of sea urchin spermatozoa.

Mercury, in contrast to zinc and to managanese and copper, had not until recently been regarded as a biologically significant constituent of seawater. Nevertheless this heavy metal ion is becoming increasingly important as a pollutant. We found *Arbacia* sperm cells to be extremely sensitive in both dose-dependent and time-dependent manner to supplements of mercury. Addition of mercuric chloride to 5×10^{-6} M (or 3×10^{8} molecules of mercury salt/spermatozoon at a working dilution of 10^{7} sperm/ml seawater), accelerates the sperm by about 170%. This increase, however, is only transitory and the mercuric ion evinces its ultimate toxicity by causing the abrupt fall in motile activity that rapidly supersedes the initial rise. For example, prolonging the incubation period from 8 to 24 minutes in 5×10^{-6} M mercury causes about a 200% decrease in the swimming speed of the sea urchin sperm from 170% above control values after 8 minutes incubation, to 29% above control values after 16 minutes incubation, to 33% below control values after 24 minutes incubation in mercury. Barron *et al.* (1948) observed that low concentrations of mercury (5×10^{-6} M) caused an 88% increase in the respiratory activity of *Arbacia punctulata* spermatozoa while higher concentrations of mercury (10^{-4} M) caused complete inhibition of the respiration. This they attributed to the fact that low levels of mercury, in combining with soluble —SH

groups that are important in the regulation of cell respiration, abolish that regulatory function, while with further increases, the mercury next combines with fixed —SH groups on essential proteins to inhibit enzymatic activity. Although we observed that low concentrations of mercury (5×10^{-6} M) caused an initial large increase in motile activity, we were still able to record motile activity in spermatozoa suspended in seawater containing mercury concentrations as high as 10^{-4} M. When exposed to this concentration of mercuric ion, the sperm swam at 133% of control rate after 8 minutes, at 54% of control after 16 minutes, and at 41% of control after 24 minutes exposure.

Nevertheless some critical amount of certain of the heavy metal ions must be necessary for optimum motility since EDTA drastically reduces the swimming speed of dilute suspensions of *Arbacia* spermatozoa. The effect of EDTA in increasing the life span and fertilizing capacity of sea urchin spermatozoa by removing trace metals from seawater has been discussed by several investigators (Rothschild and Tyler, 1954; Mohri, 1956; Tyler, 1953). Rothschild and Tyler (1954) calculate that one micromole/liter of EDTA is sufficient to bind all the trace metal ions in ordinary seawater. In our experiments, 10^{-6} M EDTA had no effect on the swimming speed of *Arbacia* spermatozoa. However, the spermatozoa used in this study were in dilute suspension and it is probable that low steady state conditions already prevailed even though we completed our first measurements within 5 minutes of dilution. Decreases in swimming speed did not become apparent until the EDTA was raised to 2×10^{-4} M/l. At this concentration motility was 83% of control values while in 8×10^{-4} M EDTA, motility further decreased to 13% of control values. Since EDTA did not affect *Arbacia* sperm swim speed except at high concentrations and since the concentration range of its effectiveness was sharply limited, it is possible that EDTA brought about a decrease in sperm motility by interfering with transport of divalent cations from cell surface sites as well as by removing trace metals from the seawater.

In this paper we have shown that, although a critical level of certain heavy metal ions must be maintained for optimum sea urchin sperm motility, an excess of these ions in the seawater adversely affects the propulsive activity of the spermatozoa. While metal ions can induce changes in motility, the mechanism of their effects on specific sperm enzymes and on the fertilizing capacity of the sea urchin spermatozoa remains to be investigated. In any case, as a probable consequence of the accumulation of heavy metals as pollutants in our oceans, large deficits in the reproductive capabilities of marine invertebrates will begin to become more apparent.

Summary

Optimum motility of sea urchin spermatozoa for a period adequate to initiate the process of fertilization requires an apparently critical level of certain heavy metal ions. Increase of some of the divalent cations above the "normal" seawater content accelerates or depresses the swimming speed in dose- or time-dependent fashion (or both). The different patterns of *Arbacia* sperm swimming speed response to the individual cation supplements (Cu, Zn, Mn, Hg) may reflect differences in rate of penetration into the cell, binding of atcive groups or selective inhibition of as yet unspecified enzymes at or below the cell surface which directly

or indirectly contribute to regulation of flagellar contractility. The concentrations tested ranged from 500 nM up to 10 mM. The "optimum" concentrations fell between 1 to 10 micromolar Cu, Zn and Hg on initial exposure, while Mn was moderately inhibitory at these levels. EDTA, up to 10^{-4} molar, exerts no adverse effect on sperm propulsion, while 8×10^{-4} M almost completely blocks the motility. Within this short concentration span, the EDTA appears not only to bind essential seawater cations, but may also deplete those intracellular regulatory cations which otherwise may exist in a state of dynamic equilibrium with the seawater.

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